S/N 09/603,448 PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	THOMAS ET AL.	Examiner:	J. FREDMAN
Serial No.:	09/603,448	Group Art Unit:	1655
Filed:	JUNE 26, 2000	Docket No.:	10552.26US01
Title:	CYTOTOXICITY TESTING		

CERTIFICATE UNDER 37 CFR 1.8: The undersigned hereby certifies that this Transmittal Letter and the paper, as described herein, are being sent via facsimile transmission to the addressed person at: ATTN: Examiner Jeff Fredman, Commissioner for Patents, Washington, D.C. 20231, fax number (703) 305-3014, on November 16, 2001.

By: Sheryl A. Boerboom
Sheryl A. Boerboom

COMMUNICATION

Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Please include the enclosed Declaration under 37 C.F.R. § 1.131 and its appended laboratory notebook pages as support for the Amendment and Response mailed on October 25, 2001. The Amendment stated that the Declaration and its supporting documents would be provided in conjunction therewith. However, these documents were not included and are now provided. Consideration of the enclosed documentation in conjunction with the Amendment and Response is appreciated.

The Examiner is invited to contact applicant's undersigned representative at the telephone number listed below, if the Examiner believes that doing so will expedite prosecution of this patent application.

Respectfully submitted,

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Dated: Nov 16, 2001

By Mark T. Skoog
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Reg. No.: 40,178

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A Professional Corporation

Fax Transmission | November 16, 2001

TO: Commissioner for Patents
Attn: Examiner Jeff Fredman
Patent Examining Corps
Facsimile Center
Washington, D.C. 20231

FROM: Mark T. Skoog

OUR REF: 10552.26US01
TELEPHONE: 612.371.5240

Total pages, including cover letter: ~~22~~ 23

PTO FAX NUMBER 1-703.305.3014

If you do NOT receive all of the pages, please telephone us at 612.371.5240, or fax us at 612.332.9081.

Title of Document Transmitted: Communication and Declaration Under 37 C.F.R. 1.131

Applicant:	THOMAS ET AL.
Serial No.:	09/603,448
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Group Art Unit:	1655
Our Ref. No.:	10552.26US01

Please charge any additional fees or credit overpayment to Deposit Account No. 13-2725. Please consider this a PETITION FOR EXTENSION OF TIME for a sufficient number of months to enter these papers, if appropriate.

By: Mark T. Skoog
Name: Mark T. Skoog
Reg. No.: 40,178

I hereby certify that this paper is being transmitted by facsimile to the U.S. Patent and Trademark Office on the date shown below.

November 16, 2001

Cheryl A. Boerboom
Signature

November 16, 2001
Date

GEN033.DOT

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By: Sheryl A. Boerboom
Sheryl A. Boerboom

DECLARATION UNDER 37 C.F.R. § 1.131

Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Susan M. Thomas, declare and state the following:

1. I am the inventor of the subject matter of the patent application identified above. I carried on my inventive activity as a faculty member at Flinders University.

2. I understand that the Examiner has cited the *Justus et al.* (*Mutagenesis* (1999) 14(4):351-6) as prior art in prosecution of the application identified above. I understand that the *Justus et al.* reference was published in an issue of *Mutagenesis* dated July 1999.

3. I further understand that the original filing date of my present patent application Serial No. 09/603,448 is June 26, 2000.

4. I state that before the publication date of the *Justus et al.* reference, that is before July 1999, I invented the subject matter described and claimed in the patent application identified above. As evidence, please find accompanying this declaration a photocopy of a report from my laboratory documenting at least the conception of the claimed invention before July 1999. I then diligently proceeded with implementing the present invention and filing the present application.

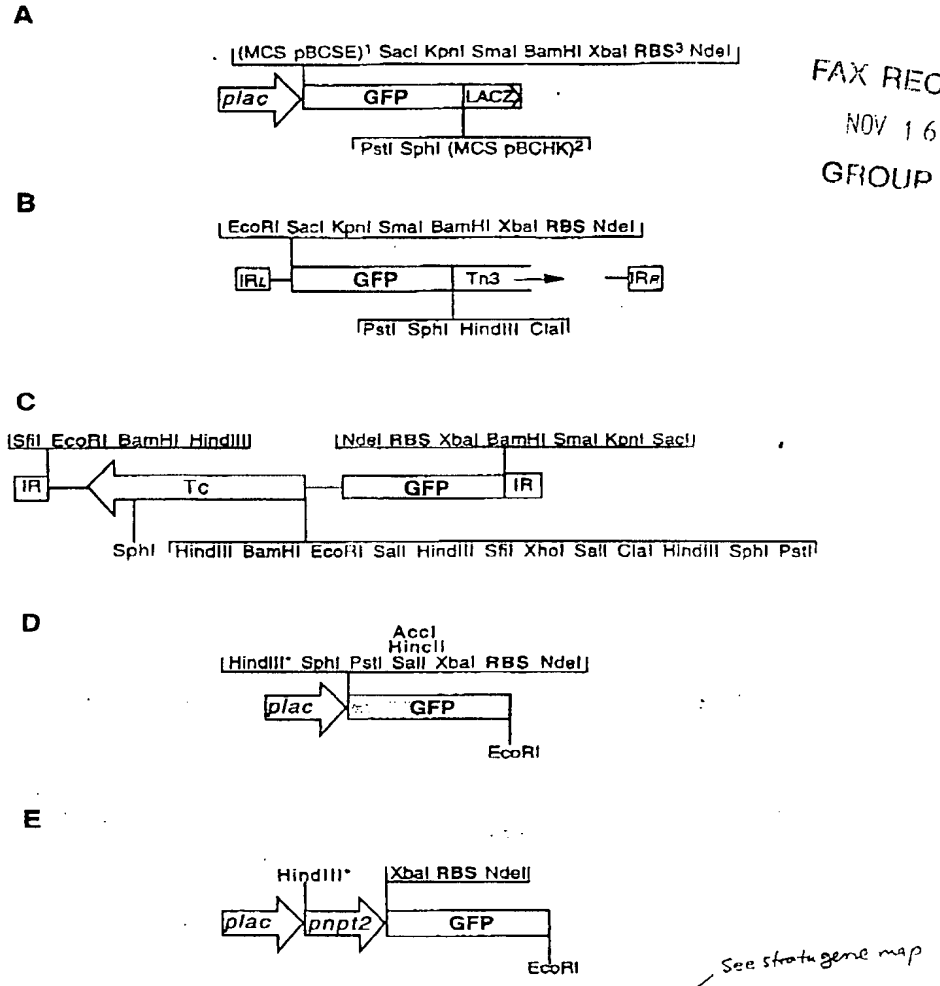
5. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that the statements are made with the knowledge that willful false statements and the like are

punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

5 Oct 2001
Date

Susan M. Thomas

*2355
?*



See stratagene map

Fig. 1. Restriction enzyme maps of *gfp* constructs. (A) pBC*gfp*; [1]multiple cloning site (MCS) from pBC SK+ from *Sac*I to *Eco*RI inclusive, [2]multiple cloning site from pBC SK+ from *Hin*dIII to *Kpn*I inclusive, [3]ribosome binding site (RBS). (B) Tn*3gfp*; the right end of pTnJHoHoI from the *Cla*I site to the right inverted repeat (IR_R) is unchanged, and includes the *bla* gene and a *Sac*I site. (C) pUTmini-Tn*5gfp*; *lux* in pUTmini-Tn*5luxAB* was replaced by *gfp*. (D) p519*gfp*, *gfp* replaced part of the polylinker downstream of *plac* in pDSK519; *there is a second *Hin*dIII site in the Nm^R gene in the vector. (E) p519*nptgfp*, *pnpt2* was inserted between the *Hin*dIII and *Xba*I sites in front of *gfp* in p519*gfp*. Diagrams are not to scale and only show altered parts of the vectors.

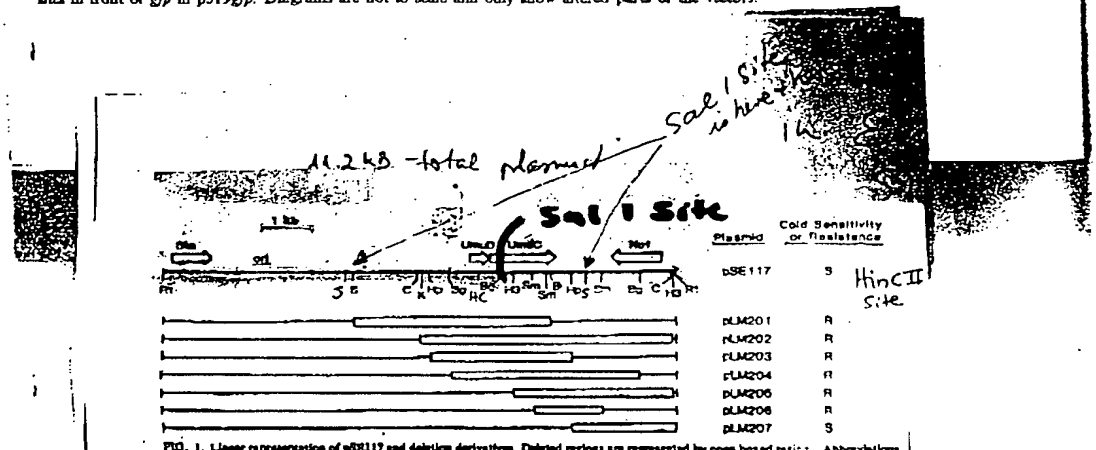


FIG. 1. Linear representation of pSE117 and deletion derivatives. Deleted regions are represented by open boxed regions. Abbreviations for restriction sites are as follows: *Eco*RI, RI; *Bam*HI, B; ClaI, C; *Kpn*I, K; *Hpa*I, Hp; *Bgl*II, Bg; *Hin*dIII, HJ; *Sma*I, Sm. Also shown are the origin of replication, *ori*; ampicillin phosphotransferase gene (transposon resistance), Ptn; and β-lactamase gene (ampicillin resistance), Bla. Deletions have the following endpoints: pLM201; *Bam*HI–*Bam*HI; pLM202, *Cla*I–*Cla*I; pLM203, *Hpa*I–*Hpa*I; pLM204, *Bgl*II–*Bgl*II; pLM205, *Hin*dIII–*Hin*dIII; pLM206, *Sma*I–*Sma*I; pLM207, *Hpa*I–*Eco*RI. The ability of each plasmid to confer cold sensitivity on KM1190 was determined by streaking the plasmid-bearing KM1190 strains at 42.5°C. In each case cold-sensitive strains were <0.1% viable at 30°C; resistant transformants yielded more than 50% viable at 30°C. kb, Kilobase.

GFP - cloning into PSE117

CLONING VECTORS

PECIAL FEATURES
2.96-kb colony-producing phagemid
High copy number ColE1-based phagemid
Large and versatile polylinker in two orientations
f1 origin also available in either orientation
T3 and T7 promoters contains α*lacZ*

PPLICATIONS
High-resolution restriction mapping
Creation of exo/mung nested deletions
Single-stranded rescue
Double- and single-stranded sequencing
In vitro RNA transcription

LONING SITES
21 unique restriction sites in multiple cloning region

ELECTION Blue/white color selection

CREENING By prokaryotic expression with antibodies or nucleic acid probes

RANSCRIPTION/EXPRESSION
In vitro RNA transcription with T3 or T7 RNA polymerase
Expression of fusion proteins

UE/WHITE COLOR SELECTION
ectors containing a portion of the *lac*Z gene provide α-complementation when ated on cells containing *lacZΔM15* on the F′. When no insert is present, a nctional α-peptide is produced that complements the gene product of *lacZΔM15* produce a functional β-galactosidase protein. When plated on indicator plates ntaining IPTG and X-gal, the colonies are blue. When a cloned insert interrupts e *lac*Z α-peptide, no complementation occurs and colonies appear white.

SION PROTEIN EXPRESSION
n inducible *lac* promoter upstream from a *lacZ* gene allows the production of sion protein. Plasmid clones may then be screened with antibody probes.

VITRO RNA TRANSCRIPTION/HIGH-RESOLUTION RESTRICTION MAPPING
ectors containing T3 and T7 bacteriophage promoters allow efficient *in vitro* thesis of strand-specific RNA. BssH II sites flanking the T3 and T7 promoters low isolation of a cassette containing the insert and the two promoters. High-olution restriction maps can then be generated with T3 and T7 primers using ratagene's FLASH[2] nonradioactive gene mapping kit.

ONUCLEASE III/MUNG BEAN DELETIONS
e pBluescript® II vector's 21 unique restriction sites within the polylinker are ifically ordered to allow production of nested deletions using exonuclease III d mung bean nuclease.

DNA RESCUE
ctors containing the f1 origin of replication from the f1 filamentous phage low rescue of single-stranded DNA upon co-infection with helper phage. The + and (-) orientations of the f1 intergenic region allow the rescue of either the ense or antisense DNA strand. Single-stranded DNA can then be used for se-ncing or site-directed mutagenesis.

ers
T7 22-mer, M13 (-20), M13 reverse, SK 20-mer and KS 17-mer (see
refer to the table on page 38 for a complete list of this vector's properties
the Appendix for the vector maps.

ts
II phagemid vector
MRF

ces
Acids Res. 16, 7583–7600.
(1988) Nucleic Acids Res. 17, 9494.

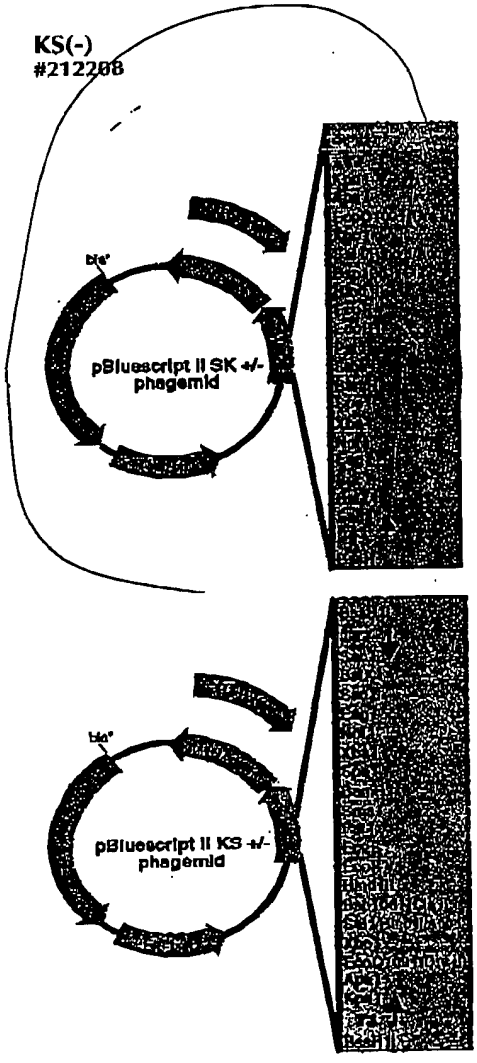
pBluescript® II Phagemid Kits

SK(+)
#212205

SK(-)
#212206

KS(+)
#212207

KS(-)
#212208



gfp - cloning into PSE117

Have decided to clone gfp into same site as this gene is not effected by metabolic conditions like hux is. The last few experiments - Spent broth induction
- temp " have given variable or no solid result due to possible interference with hux - this thus doesn't reflect true umu gene induction.

INSERT

I have obtained a gfp PCR product from

I want to clone gfp with Hind 3, EcoRI ends ∴ use D (this is the fragment has given me. However its ends are blunt so it needs to be cut with Hind3 and Eco RI at either end.

Vector

Cut pSE117 Hind 3 isolate from gel ~7kb cut end with Eco RI to give one end Hind 3 other Eco RI then ligate gfp to this vector.

Run on a gel 2 µl of Sample eek concentration.

got 2 bands should have one ~700bp fragment need to PCR to make sure I have got the correct fragment.

TEST KUN PCR

Set up as follows:- n = 2

+ve control	-ve control.
1 µl DNA	1 µl H_2O (on bench
1 µl dNTPs 10mM 4 µl Mg 25mM (final 2mM) 5 µl x10 buffer 2.8 µl primer gfp HindIII (25pmol each) 2.3 µl primer gfp EcoRI 1 µl Taq polymerase (1 Unit) 32.9 µl H_2O 50 µl total volume.	the same as +ve control

- make bulk mix
- Split in 2 49 µl ⊖ + 49 µl ⊕
- and add mineral oil - run in thermocycler ~3 hours

94°C	30S	
47°C	30S	30 cycles
72°C	2 min	1 cycle
72°C	10 min	
28°C	5min	

low stringency due to using plasmid DNA template and specific primers. If using genomic DNA need higher specificity ∴ increase this temp.

Lane:- 1 1 µl marker
2 5 µl + pcr reaction) 5 H_2O
3 5 µl - control 5 H_2O

looks o.k. ~ 700 bp fragment, ⊖ control - no DNA which is good.

PCR

2 tubes
n=2

on bench

- 1 µl H_2O — ⊖
- 1 µl DNA — ⊕

controls

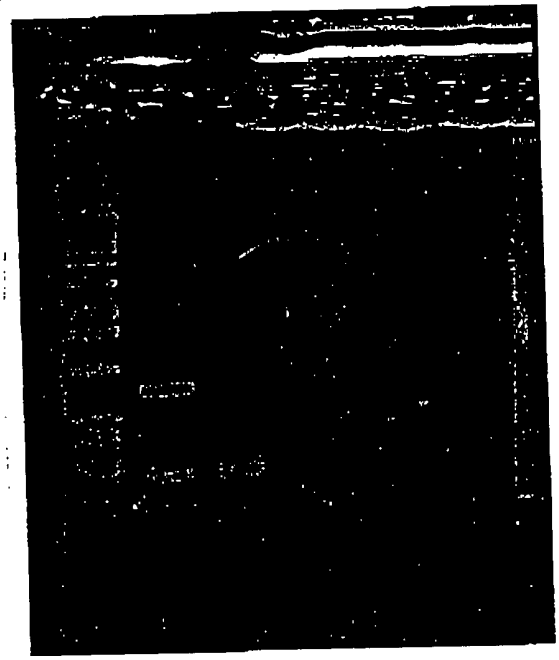
in cabinet

2	dNTP's	1 µl
8	Mg	4 µl
10	X10	5 µl
5.6	PCR HindIII	2.8 µl
4.6	PCR Eco	2.3 µl
2	~~Taq~~ poly	1 µl
65.8	dH2O	32.9

49 µl → + control

49 µl → − control

2 wells



Scaled up version

- ⊖ controls ⊕

- Run 3 x 100 µl reactions. 1 x Som
- Set up the same cycles + steps as for +

3 tubes.
2 µl each
(6 µl total)
BULK MIX

1 tube
1 µl
control H_2O

7 µl Dntps
28 µl Mg
35 µl 10x
19.6 µl Hind (P)
16.1 µl Eco (P)
3.5 µl Taq
233.8 µl H_2O

98 µl bulkmix per tube.
294 µl
343 µl total bulkmix for 4 tubes

add 49 µl bulkmix

Run gel :- lane 1 :- 1 µl marker DNA.
2 :- reaction 1 (3 µl) +
3 :- reaction 2 (3 µl) +
4 :- reaction 3 (3 µl) +
5 :- control reaction (3 µ

mini gel 1% 100 V

DNA looks good + control has worked

- pool all 3 preps + run through colun to remove primers.

POK

2 min 94°C CYCLE 1

steps

30" sec 94°C 1 CYCLE 2

30" sec 47°C 2 ↓

2 min 72°C 3

31

10 min 72°C 32 cycle 3

33 cycle 4

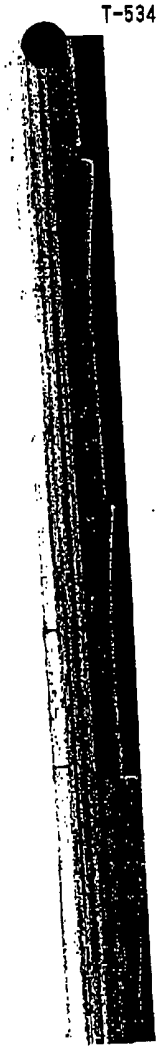
0 25°

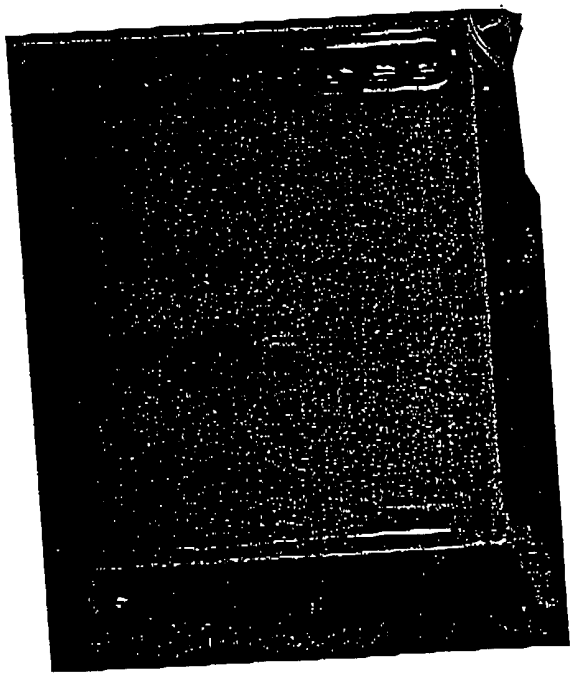
Steps

Ramp Rate

NC

Run- prog 1





26

pooled DNA → ~~≈~~ 300 ul without mineral oil
avoid

Extracted once with equal volume chloroform : isoamyl alc
24 : 1

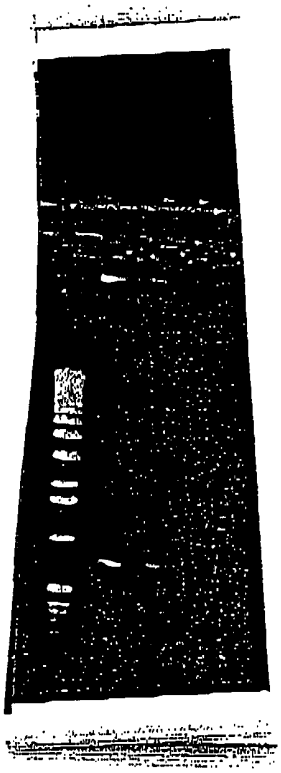
(looked milky)

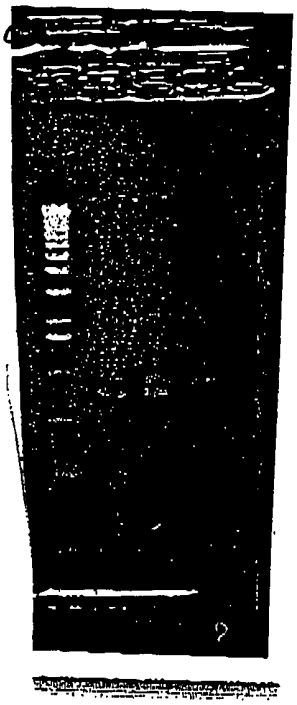
- Run supernatant through promega PCR clean up prep column. as per manufacturers protocol except evaporate off ~~isoamylalco~~ isopropanol at 37°C for 15min.
- elute DNA in 2x 50 ul TE

100 ul Total volume

Run 1 ul on gel to check.
of 100 ul total lane 2
Lane 3 Serinas GFP 2 ul.

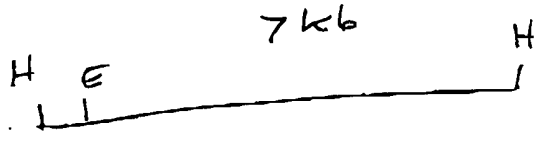
DNA looks ok. appears there
may also be a small band above?
not sure what this is it has occured in
prep also but at a larger quantity.





CLONING STRATEGY

Digestion of vector + Insert see pge 22



pSE117

↓ digest EcoRI

7kb.

from plasmid pBCgfp using Hind3 + EcoRI primers

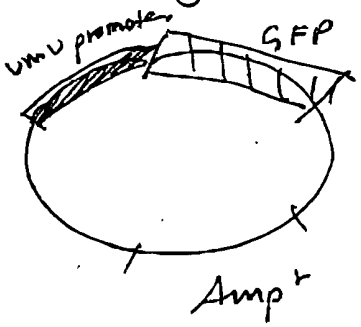
GFP PCR product

↓ digest HindB EcoRI.

740bp

↓

ligate + transform into E. coli GW2100



new plasmid. construct using gfp.

no need for alkaline phosphatase treatment as vector cannot religate to itself. it has 2 different ends.

Insert must go in in one orientation because 2 different ends.

- Run preps from digest through the wizard clean up columns as per usual protocol. 50 μl TE total

- checked on gel. lane 1:- 1 μl marker
2:- 5 μl digest 1 700bp GFP CK
3:- 5 μl digest 2 pSE717 7Kb

don't know why more than one band column may break up? DNA?
go ahead + ligate anyway use 5 μl of each looks about equal should be enough

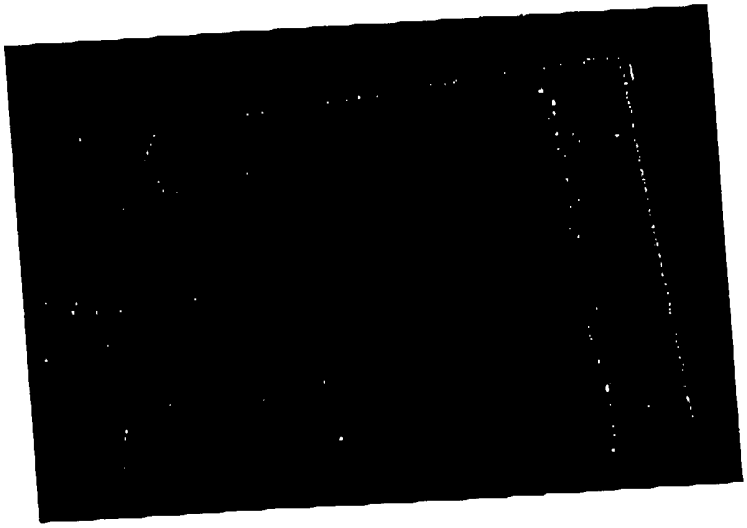
Ligations [1:1 Ratio]

5 μl vector ✓
5 μl insert (GFP) ✓
~~μl H_2O~~?
1 μl 10 mM rATP (Stratagene.
1 μl 10× ligase buffer ✓
1 μl T4 DNA ligase

- 16°C o/n

TRANSFORMATIONS

- Transformed E. coli GW2100 as per usual method using N. broth. added all of ligated mix plated out onto (Amp 50 μg/ml) all of cells in 1 ml growth from flask.
37°C o/n



RESULTS GFP cloning

got about 6 colonies from the whole transformed mixture when plated out along with patches of growth ⇒ may be due to cells feeding on dead cells. 20 patches. I streaked out these colonies and the patches onto Amp agar. 37°C o/n

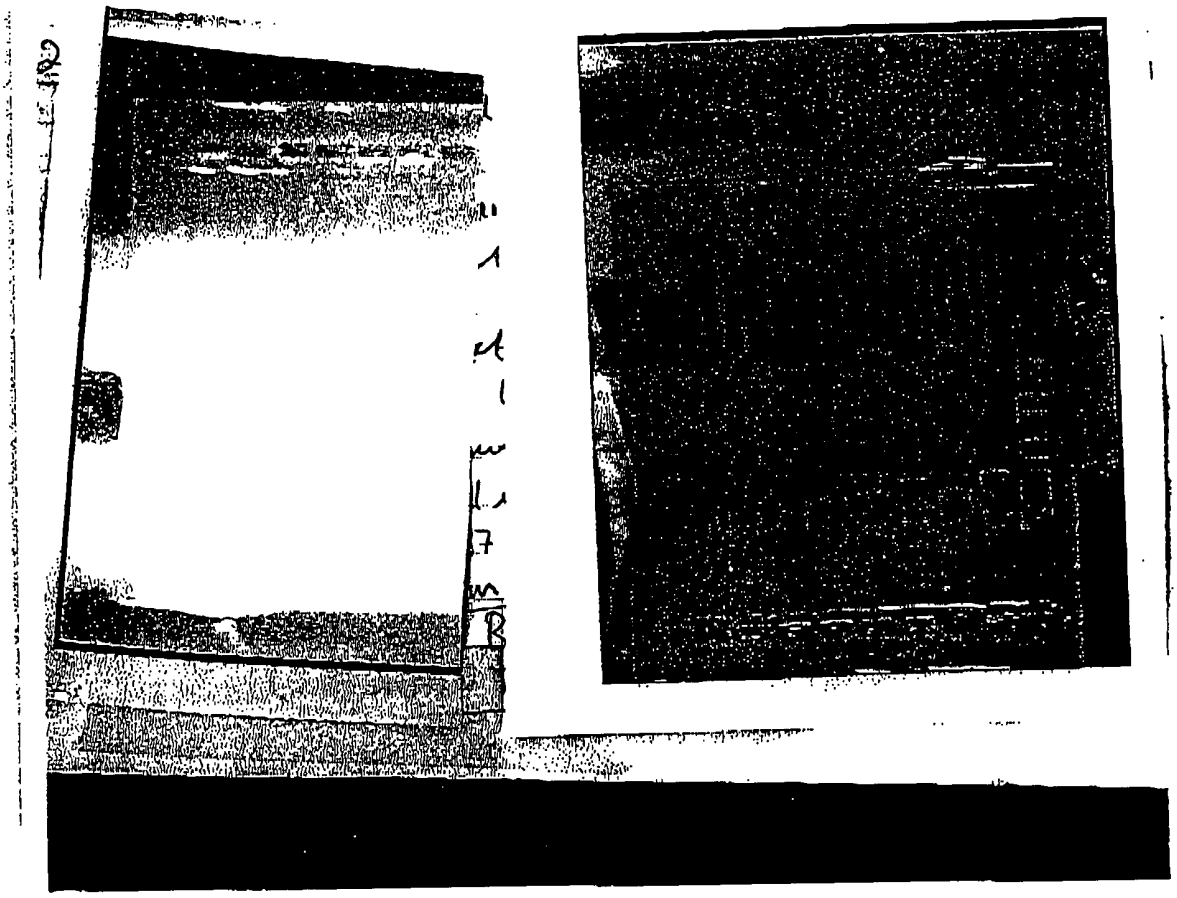
Colonies 1, 3, 9, and 12 have grown up when streated. all the rest no growth. Hopefully these are o.k. it is possible that they may be incompletely digested vector which has re ligated to itself! Need to do plasmid preps to check this out.

Set up 10 ml sam/ml Amp N.B. cultures 1, 3, 9, 12 37°C o/n

— No. 1 didn't grow — all rest did.

did rapid miniplasmid prep as per usual. 50 ul TE

1 :- 1 ul marker
2 :- 5 ul uncut No. 2, 5 ul H_2O
3 :- 5 ul 2 cut 1 ul EcoRI 1 ul Hind 3, 1 ul Buffer B,
4 :- 5 ul uncut No. 3, 5 ul H_2O
5 :- 5 ul 3 cut 1 ul EcoRI 1 ul Hind 3 1 ul Buffer B
6 :- 5 ul uncut No. 4, 5 ul H_2O
7 :- 5 ul 4 cut 1 ul EcoRI, 1 ul Hind 3, 1 ul Buffer B
8 :- 4 ul pSE117 7kb fragment EcoRI Hind3 digested. 22.3.97 6 ul H_2O
10+9 :- Russels samples.
1 1 1 ul BSA to each digest
2 1 ul [illegible]



30

DISCUSSION

Results on gel pge 29 look good. There is a ~ 700bp fragment in lane 3 and 5. so gfp 2 and gfp 3 both h cloned gfp gene in pSE174.

Hind
EcoRI

gfp 2		gfp 3		gfp 4	
obs.	exp.	obs.	exp.	obs	exp.
7kb	7kb	7kb	7kb	~~7kb 3kb~~	7kb
700bp	700bp	700bp	700bp	~~700bp~~	700bp

gfp 4 — not sure about this one uncut ~~cut~~ bands ~~dont~~ do not match with ex fragments? need to re-check

Now need to check if UV and mutagens in gfp production. and compare against the gfp construct.

Did a plasmid prep of gfp 1 to check if Gfp p

digest 37°C for a few hours — 1 incubation,
1) (cut) 4µl DNA, 1µl Eco 1µl Hind 3 1µl Buffer
2) (uncut) 4µl DNA, 5µl H_2O

gfp model

FLOVROMETER DETECTION

Set up o/n 37°c cultures gfp 1→4
50 mg/ml Amp

cultures 1, 2, + 4 grew but not 3?

- Spin down culture gfp 1 as this looked good a gel
- diluted + resuspended in phosphate buffer 20mls.
- ~~Test~~ UV irradiated 3mls 20sec 2nd shot
  NO UV ~~is~~ 3mls
- 1ml → flouromiter curette of each
- blank machine using 0 UV dose tube to zero
- then get a reading of induced culture.
- Tested No. 2 because from gel this one has insert No1 didnt know yet No. 4 didnt have insert.

No2 Results

Time
10min -
30min - 190
1 hour - 200
2 hour - 170

DISCUSSION

- need to use log-phase cells as signal seem to be very low. Couldnt really see much under microscope either Redo using log phase cells.

MACHINE STUFFED
NEED TO REDO - need to test all clones

- only has the following filters
  440 nm
  665nm
  530nm.

32

- also spotted culture onto microscope slide and sealed coverslip with nail polish
- check using
- can see faint cells at 15 min need longer check 30 min-45min later

Tested gfp 2 only

= See discussion page 31

Set up o/n culture gfp 2 +3 (fluorescence looks good) pge 29 gel

---

UV reading = 10.10 = 15 Seconds UV

Tested No. 2 No response even after 4-5

Checked No. 3 after this time and it was fluorescing! on micro slide

Need to Retest No. 3, 4 and 1 the same way

Took some photos on Camera. Photos turned out o.k. See photo Album

Need to check all as not all appear to do this even though they seem to have appropriate gene insert